

Different MHC Class I Alleles Compete for Presentation of Overlapping Viral Epitopes

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Summary

We previously identified an HLA-B8⁺ donor, NW, whose lymphoblastoid cells failed to present a B8-restricted epitope from the influenza A nucleoprotein following viral infection, although added peptide could still be presented. The failure to present through HLA-B8 following viral infection appears to be specific for the NP epitope. Here, we report that donor NW makes an HLA-B2702-restricted influenza-specific CTL response to an epitope in the nucleoprotein that overlaps the B8-restricted epitope by 8 aa. Two mechanisms for the failure of this cell line to present the B8-restricted epitope following viral infection are investigated. One is that there is an antigen processing polymorphism specific to the NW cell line, so that there is either preferential generation or preferential transport of the B2702 epitope. The other is that B8 and B2702 compete for a common peptide fragment in the ER and this leads to suboptimal loading of HLA-B8.

Introduction

Major histocompatibility complex (MHC) class I molecules bind peptides, derived from intracellular protein pools, and display them at the cell surface for recognition by CD8⁺ cytotoxic T cells (Townsend and Bodmer, 1989). These peptides are generally 8–11 aa long and, as revealed by X-ray diffraction studies, are an integral part of the class I structure (Bjorkman et al., 1987; Garrett et al., 1989). The peptide binding site is a groove formed by two α helices lying across an eight-stranded β sheet and it is the fine structure of this groove that determines the nature of peptides bound (Madden et al., 1991).

Although class I molecules associate with their peptides in the endoplasmic reticulum (ER), they actually recruit these peptides from the cytosol via an active transport process that utilizes the TAP complex. This complex is a heterodimer consisting of the TAP1 and TAP2 subunits

and is intimately involved in the assembly of class I- β 2-peptide complexes. Recent studies have shown that TAP not only preferentially transports peptides of appropriate length for class I binding (Androlewicz et al., 1993; Neefjes et al., 1993; Shepherd et al., 1993), it actually facilitates such binding by physically associating with class I- β 2 dimers as they assemble in the ER (Ortmann et al., 1994; Suh et al., 1994). Human, mouse, and rat transporters have some peptide sequence preference, particularly regarding the N and C termini as well as the penultimate C terminal residue (Heemels et al., 1993; Momburg et al., 1994b; Androlewicz and Cresswell, 1994; Schumacher et al., 1994), but a role for the fine specificity of the transporter in antigen presentation, if any, remains to be established.

Proteolytic processing of antigenic proteins in the cytosol precedes translocation of peptides into the ER; these processes however, are still ill defined. The proteasome, a large multisubunit cytosolic protease with multiple cleavage specificities, is thought to be involved. Two proteasome β -type subunits, LMP2 and LMP7, are encoded by genes in the class II region of the MHC (Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Martinez and Monaco, 1991; Ortiz Navarrete et al., 1991). These subunits are γ interferon inducible (Ortiz Navarrete et al., 1991; Yang et al., 1992) and can alter the specificity of the proteasome (Driscoll et al., 1993; Boes et al., 1994). This has suggested a role in antigen processing and presentation for these two subunits, although transfection studies have shown that these subunits are not essential for the presentation of all antigens (Arnold et al., 1992; Momburg et al., 1992; Yewdell et al., 1994; Zhou et al., 1994).

In humans and mice, the binding specificity of the class I molecule is believed to be the primary determinant in the selection of antigenic peptides for presentation to cytotoxic T lymphocytes (CTL) (Falk et al., 1990), and usually the presence of the restricting class I molecule alone is sufficient for the appropriate processing and presentation of a given epitope (Wallny et al., 1992; Rotzschke et al., 1991; Lobigs and Mullbacher, 1993). It is, therefore, unusual for a cell line expressing the correct class I molecule to fail to present an epitope restricted by that allele. In previous studies of antigen presentation, however, we identified a donor (NW) whose lymphoblastoid cells, despite having normal cell surface expression of HLA-B8, failed to present a known B8-restricted epitope from the influenza A nucleoprotein following viral infection, although exogenously added peptide was presented normally (Rowland-Jones et al., 1993). Furthermore, the failure to present through B8 in these cells appears to be specific for the influenza nucleoprotein epitope, since at least three other B8-restricted epitopes are presented normally by these cells following viral infection (Rowland-Jones et al., 1993 S. R.-J., unpublished data).

In the present studies, we have investigated the molecular basis for the unusual properties associated with B8-restricted antigen presentation of the NW cell line. We

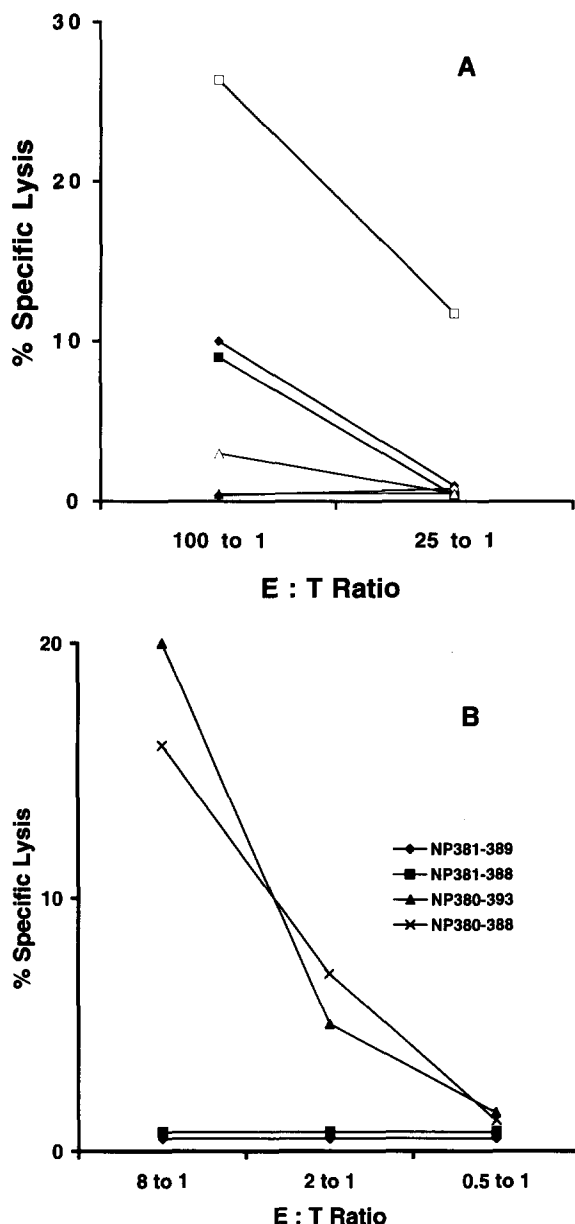


Figure 1. Donor NW Generates a B2702-Restricted Response to the Influenza A Nucleoprotein that Overlaps the B8 Epitope by 8 aa
(A) The influenza A-specific CTL response from NW is B2702 restricted and maps to NP residues 381–388. Influenza-specific bulk cultures were established from NW PBMCs using influenza A virus AX/31 and were tested on influenza-infected cells or cells incubated with peptide. Targets were either HLA matched at only B2702 (C1R-B2702) or at his other A and B alleles (HW-HLA-A3, A33, B8). Specific lysis of targets in the absence of peptide is subtracted from each value. Targets are (open square) C1R-B2702 plus NP 381–388, (closed square) C1R-B2702 plus influenza A, (closed diamond) C1R-B2702 plus NP 381–389, (closed triangle) HW plus NP 381–388, (open triangle) HW plus influenza A, (open diamond) HW plus NP 381–389.
(B) NP residue 380 is required for B8-restricted CTL recognition. Peptides were included in the assay at a final concentration of 10^{-6} M. The NW B cell line was used as a target. Effectors are CTL clone HW5 established from donor HW. Specific lysis of targets in the absence of peptide has been subtracted from each value.

380ELRSRYWAIRTR391
380ELRSRYWAI 388 HLA-B8
381LRSRYWAI388 HLA-B2702
383SRYWAIRTR391 HLA-B2705

Figure 2. Residues 380–391 of the Influenza A Nucleoprotein
Three human CTL epitopes mapping to this region are shown. The B2705 epitope maps to residues 383–391, B2702 maps to 381–388, and B8 maps to 380–388.

establish that donor NW does, in fact, make an influenza A-specific CTL response directed towards an epitope in the nucleoprotein. This response, however, is not restricted by HLA-B8 but rather by the other B allele of the donor, HLA-B2702. We have mapped the B2702 epitope and find that it corresponds to residues 381–388, while the B8 epitope corresponds to residues 380–388. Since overlapping epitopes, particularly of this extent, could present difficulties for the antigen processing and presentation machinery, this suggested possible explanations for the failure of NW to present nucleoprotein (NP) 380–388. Two possible, not necessarily mutually exclusive, mechanisms are investigated. One is that there is an antigen-processing or selection polymorphism specific to the NW cell line, so that there is either preferential generation or preferential transport of NP 381–388. The other is that as B8 and B2702 assemble in the ER they compete, at the level of binding, for a common NP peptide fragment and this leads to suboptimal loading of B8. We provide evidence for the latter of these two possibilities.

Results

The NW Influenza-Specific CTL Response Reveals a Cluster of Epitopes in the Nucleoprotein

Influenza-specific CTL were generated from donor NW following natural influenza A virus infection. These CTL recognize influenza-infected target cells that are HLA matched only at B2702 and fail to recognize infected target cells matched only at any of his other class I alleles, which include A3, A33, and B8 (Figure 1A). Experiments using B2702-matched targets and recombinant vaccinia viruses revealed that the NW CTLs were responding to an epitope in the nucleoprotein. However, this was not the previously described B2705-restricted peptide, NP 383–391. Instead, the epitope mapped to residues NP 381–388 (Figure 1A). NP 381–388 overlaps both the B8 and B2705 epitopes (Figure 2), but it is not recognized by B2705-restricted CTL (data not shown) nor is it recognized by B8-restricted CTL (see Figure 1B). Influenza-specific CTLs were generated from another donor with B2702 (LY), who is not a member of the NW family, and these CTLs also recognize the NP 381–388 peptide when presented by B2702-matched target cells, demonstrating that the presentation of this epitope is not unique to NW cells (data not shown).

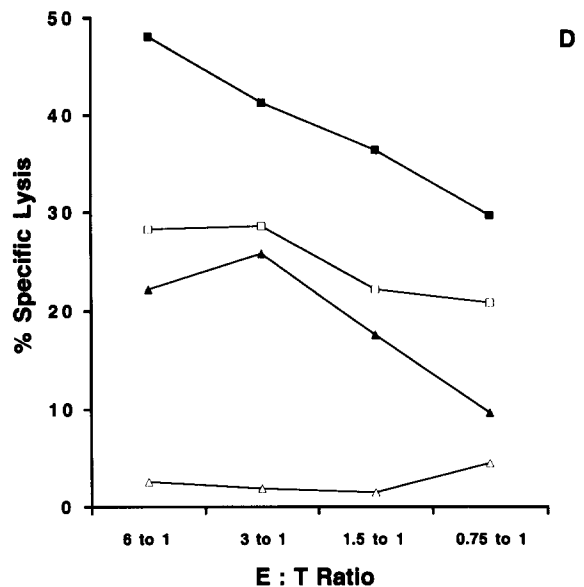
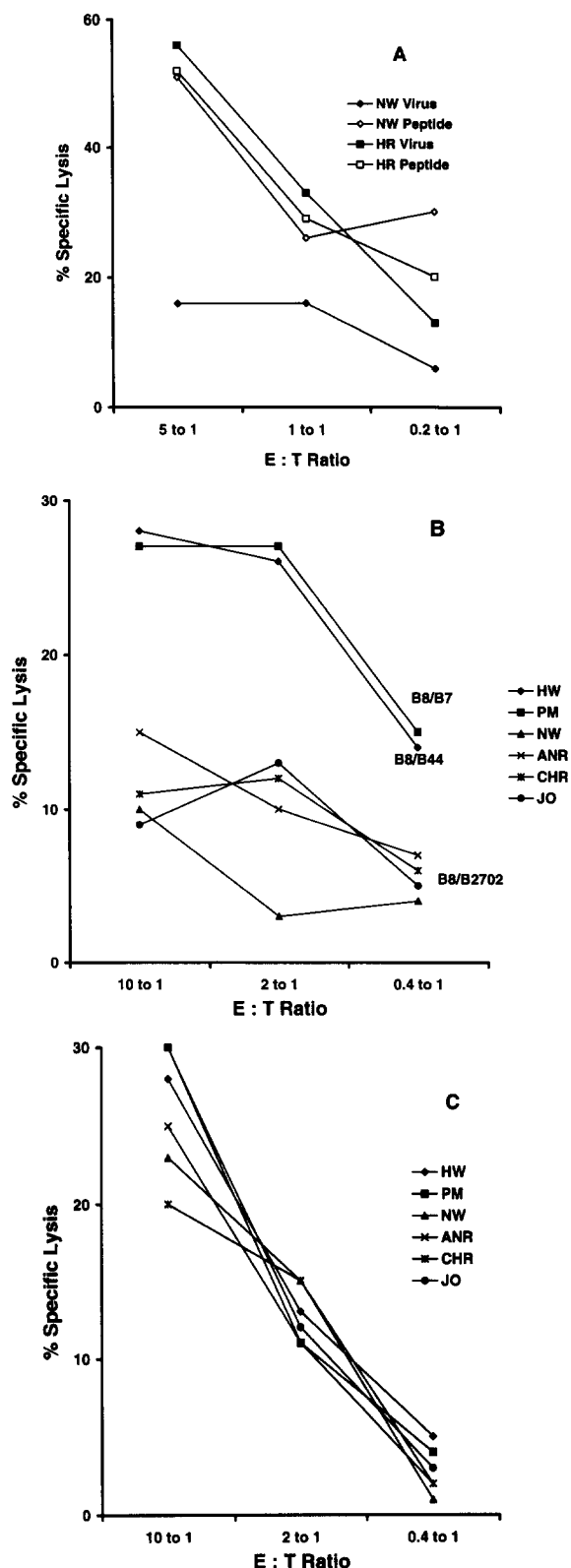


Figure 3. Cell Lines Coexpressing HLA-B8 and HLA-B2702 Fail to Present the B8-Restricted Epitope Following Viral Infection

(A) NW cells present peptide but not virus to NP-specific B8-restricted CTL. A control B-LCL, HR, which expresses HLA-B8 in combination with HLA-B7, is included for comparison. Effectors are CTL clone HR4 derived from donor HR. Targets are infected with Vac-imp. Specific lysis of targets in the absence of peptide was less than 3%.

(B) Other B-LCL-expressing B8 in combination with B2702 fail to present the B8 NP epitope following viral infection but (C) presented added peptide normally. Effector cells are CTL clone HW5 derived from donor HW and used at the E-T ratios indicated. Virally infected targets were infected with 1-2 HAU per 100 cells of influenza A AX/31 for 1 hr and allowed to express viral proteins for 2.5 hr prior to the CTL assay. For targets with peptide added, NP 380-388 was included in the assay at a concentration of 10^{-7} M. Specific lysis of targets in the absence of peptide has been subtracted from each value.

(D) C1R cells expressing the allelic combination of HLA-B8 and HLA-B2702 reproduce the NW phenotype. Effectors are a B8-restricted NP-specific CTL line established from donor HR. Targets are transfectants of C1R cells expressing either HLA-B8 (B8) or HLA-B8 and B2702 (B8-B2702) and were either infected with influenza A virus AX/31 as for (B) or incubated with peptide as for (C); (closed square) B8 plus peptide, (open square) B8 plus virus, (closed triangle) B8-B2702 plus peptide, (open triangle) B8-B2702 plus virus. Specific lysis of targets in the absence of peptide has been subtracted from each value.

Cells Expressing the Allelic Combination of HLA-B8 and HLA-B2702 Fail to Present the HLA-B8 Epitope Following Viral Infection

Figure 3A confirms the previous observation and shows

that NW cells present NP 380-388 poorly following infection with vaccinia virus expressing a truncated version of the nucleoprotein (vac-imp), despite having normal cell surface expression of B8 (Rowland-Jones et al., 1993,

L. T., unpublished data). Added peptide, however, is presented normally. In previous studies, it was established that there is not a generalized defect in antigen presentation through B8 in NW cells, since at least three other B8-restricted epitopes are presented normally following viral infection (Rowland-Jones et al., 1993). Thus, NW cells specifically fail to generate B8–NP complexes during viral infection. The overlapping B8 and B2702 epitopes in the flu nucleoprotein suggested two possible mechanisms for the failure of NW cells to present the B8 epitope following viral infection. Each mechanism depends on how epitopes are actually generated in the cell. One possibility is that the B8 and B2702 epitopes are fully processed in the cytoplasm and in NW cells there is a processing or selection polymorphism, which either leads to preferential generation or preferential transport of the B2702 epitope. Alternatively, a precursor fragment spanning both epitopes could be generated in the cytoplasm and translocated into the ER. Then, in cells where B8 and B2702 are simultaneously assembling, there could be competition for this fragment and this could lead to suboptimal loading of B8. A processing or selection polymorphism should be specific to NW cells, while the second possibility should extend to other cell lines expressing the allelic combination of B8 and B2702. We find that three other Epstein–Barr virus (EBV)-transformed B lymphoblastoid cell lines (B-LCL) coexpressing B8 and B2702 also show a marked reduction in their ability to present the epitope following viral infection but do present added peptide normally (Figures 3B and 3C). The HW (B8, B44) and PM (B8,B7) B-LCL, however, present both viral-derived antigen and added peptide normally (Figures 3B and 3C). In addition to HR (Figure 3A), HW, and PM (Figures 3B and 3C), we have tested at least five other EBV transformed B-LCL, which express B8 but not B2702, for their ability to present viral-derived antigen and find that they all present normally (Sutton, 1993; Rowland-Jones et al., 1993; S. R.-J. and L. T., unpublished data).

We also find, using transfectants of the class I-reduced human B cell line C1R, that transfectants expressing B8 in the absence of B2702 present normally following viral infection, while cells coexpressing B8 and B2702 present viral-derived antigen poorly (Figure 3D). HLA-B8 expression as judged by cell surface staining was comparable between the two transfectant cell lines; for the double transfectant, the cell surface staining of B8 relative to that of B2702 was comparable to that for NW cells (data not shown). In all experiments, the B8–B2702 transfectants presented peptide less well than the B8 transfectants. This difference, however, was not always to the extent shown in Figure 3D and was always insufficient to account for the poor presentation by virally infected targets. We conclude from these data that it is the presence of B2702 that leads to a failure to present the B8 NP epitope following viral infection. We tested the possibility that B2702–NP complexes serve as antagonists to the B8-restricted CTL and find that they do not (data not shown).

Thus, the results show that the inability to present the epitope following viral infection is not due to a processing polymorphism associated with NW cells but, rather, since

it depends on the combination of alleles in the cell, to an event occurring between the ER and the cell surface. Because the sequences of the B2702 and B8 NP epitopes are so similar, B8 and B2702 could vie for the same NP peptide fragment(s) as they are assembling in the ER. Such a fragment must contain NP residue 380, since this residue is essential for B8-restricted CTL recognition (see Figure 1B) and should extend at least through residue 388, since this residue is required for optimal B8-restricted CTL recognition (Sutton et al., 1993). Since the TAPs prefer to translocate peptides of 8–10 aa, one candidate is the fully processed B8 epitope. This peptide only represents an N-terminal extension of one for B27 and therefore may well be able to bind B2702 (see below).

NP 380–388 Can Bind B2702

The relative ability of B8 and B2702 to bind NP 380–388 was tested using a previously described cell lysate binding assay (Tussey et al., 1994). For these assays, ¹²⁵I-labeled NP 380–388 was incubated with lysates of metabolically labeled C1Rs expressing B8, B2702, or B7 as an irrelevant class I. Class I was immunoprecipitated from the lysates using the human class I-specific monoclonal antibody, W6/32. Samples were analyzed by SDS–PAGE and then quantitated using a phosphorimager (see Experimental Procedures). Representative results are shown in Figure 4A. We find that NP 380–388 binds B8 and B2702 comparably.

A Lysine Substitution at Position 382 Reduces Binding of NP 380–388 to B2702 without Altering Binding to B8

If B8 and B2702 are competing, at the level of binding, for NP 380–388 (or a slightly longer fragment) then an amino acid substitution that would reduce binding of the peptide to B27 without diminishing binding to B8 should restore B8 presentation. The sequence of NP 380–388 is ELRSRYWAI. The arginine at position 382 is an anchor for both B8 and B2702 binding. For B8, peptide elution studies have shown that while arginine is accepted, lysine is actually the preferred residue at this position (Sutton et al., 1993; DiBrino et al., 1994). Further, previous studies have shown that a lysine substitution at position 382 of the NP 380–388 peptide has no effect on either its ability to bind B8 or on CTL recognition of the peptide (Sutton et al., 1993; DiBrino et al., 1994). The peptide binding motif for both B2702 and B2705 has been determined by pool sequencing of endogenously bound peptides (Jardetzky et al., 1991; Rotzschke et al., 1994). Common to both motifs is the anchor of arginine at position 2 and virtually all of the sequenced B27-associated peptides have an arginine at this position (Engelhard, 1994). The role of the B pocket, which is identical among the B27 subtypes, in determining this anchor is well established (Madden et al., 1992). Other studies with B2705 and NP 383–391 have shown that a lysine at P2 completely abolishes presentation of this peptide, presumably as a result of a loss in binding (Colbert et al., 1993). As a first step in testing whether we could restore B8 presentation by reducing the ability of B2702 to bind the peptide, we synthesized NP 380–388 (K) (which has a lysine substitution at residue

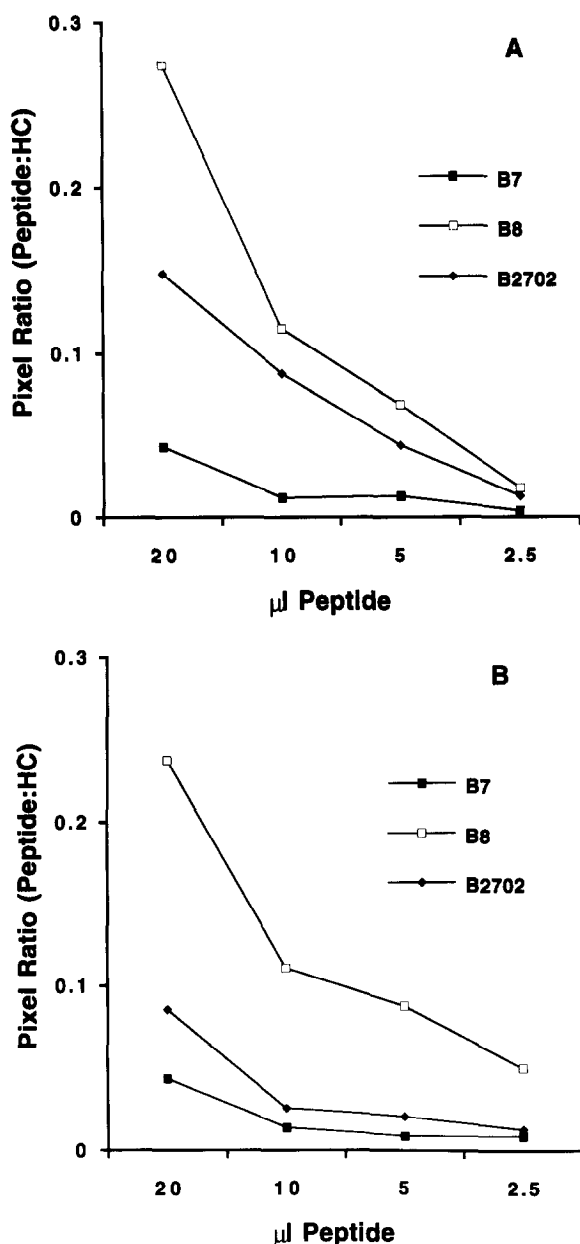


Figure 4. NP 380-388 Binds B2702 in In Vitro Binding Assays
Binding of 125 I-labeled (A) NP 380-388 or (B) NP 380-388 (K) to B8, B2702, or B7 in lysates of C1Rs expressing B8, B2702, or B7. Gels were quantitated using a phosphorimager. For each gel, pixels contained within the peptide band were normalized to pixels contained in the heavy chain band and this ratio was plotted against peptide concentration. The graphs shown represent the average of two gels and the pixel ratios were within 10%–11% between these gels. The final concentration of peptide for each point is estimated to range from 50 nM to 2 μ M (see Experimental Procedures).

382) and tested the effect of this substitution on binding of the peptide to B8 and B2702 using the same assay as for Figure 4A (Figure 4B). As predicted by the binding specificities of the two molecules, the lysine substitution at position 382 has no deleterious effect on binding to B8, while binding to B2702 is greatly diminished by this substitution.

The association of B8 or B2702 with NP 380-388 or NP 380-388 (K) following their translocation into the ER was also investigated. For these assays, streptolysin O-permeabilized cells were used as a means of introducing the peptide into the cytosol (Androlewicz et al., 1993). 125 I-labeled peptide, either NP 380-388 or NP 380-388 (K), was introduced into the cytosol of C1R cells expressing B8, B2702, or B7 as an irrelevant class I molecule. Following translocation, cells were solubilized and the class I immunoprecipitated using W6/32. The level of bound peptide was determined by counting in a γ counter. The results with the C1R transfectants show that NP 380-388 binds B2702 and B8 comparably following its translocation into the ER. The lysine substitution at position 382, however, alters the relative ability of the peptide to bind these alleles following its translocation into the ER (Figure 5A). These assays are a function of the ability of the peptide to compete for binding to class I as well as its ability to compete for translocation. Thus, the lysine substitution could affect the efficiency of translocation or the ability of the peptide to bind class I, or both. To distinguish between these possibilities, the relative ability of NP 380-388 and NP 380-388 (K) to compete for translocation was measured directly using the recently described method of Neefjes et al. (1993) and others (Androlewicz and Cresswell, 1994) and was found to be the same (Figure 5B). Thus, the differences in intracellular loading of B8 and B2702 with these two peptides are due to differences in the relative ability of the peptides to bind to HLA following their translocation into the ER. NW cells were included in these assays, but the antibody used will precipitate all class I from the cell; B8- and B2702-specific monoclonal antibodies, which are also suitable for immunoprecipitations, are not available. The counts recovered from NW were approximately the sum of the counts from C1R B8 and C1R B2702 for both peptides, so the ratio of peptide binding to B8 versus B2702 in C1Rs might actually reflect the distribution of the peptide between B8 and B2702 in NW cells: that is, NP 380-388 is evenly distributed between B8 and B2702, whereas NP 380-388(K) is preferentially associated with B8. When one compares the absolute number of counts recovered in these assays, it would suggest that the results are slightly different from those with the lysate binding assays (see Figures 4A and 4B), in that the lysine substitution appears to enhance binding of the peptide to B8. However, if one compares relative binding (i.e., B27 versus B8) for the two assays the results are actually quite similar. For example, B2702 binding of NP 380-388 is roughly 60%–70% that of B8 in the lysate binding assays, whereas it is 69% in the intracellular transport assays. B2702 binding of NP 380-388 (K) is 10%–15% that of B8 in the lysate binding assay and 28% that of B8 in the intracellular transport assay. However, because we were looking for a substitution that reduced the ability of B2702 to bind without altering B8 binding, we further tested the effects of this substitution on B8 binding using a third binding assay previously described by Gao et al. (1994). Unfortunately, this assay could not be used for B2702, which is extremely difficult to fold in vitro, but the results with B8 clearly and reproducibly show that the lysine substitution has no effect

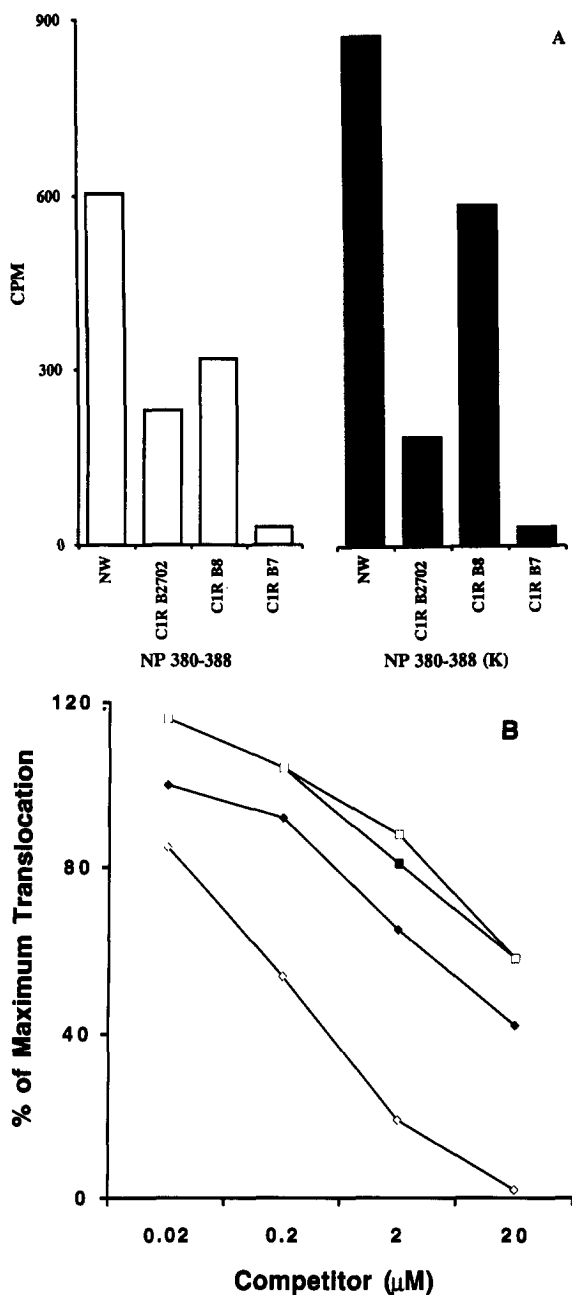


Figure 5. NP 380-388 Can Associate with B2702 Following Its Translocation into the ER

(A) Intracellular loading of ¹²⁵I-labeled NP 380-388 (open bars) or NP 380-388 (K) (closed bars) in permeabilized cells of either NW or C1R8 expressing B8, B2702, or B7. Counts represent the average of duplicates, which were within 10% of each other.

(B) The lysine substitution does not affect the ability of the NP peptide to compete for translocation. Competition curves for (closed square) NP 380-388, (open square) NP 380-388 (K), and (closed diamond) NP 381-388 are shown. The previously characterized B27#3 self-peptide (open diamond) is included as a reference peptide. The percentage of maximum translocation (y-axis) indicates the amount of ¹²⁵I-B27#3* translocated into the ER as measured by binding to concanavalin A-Sepharose.

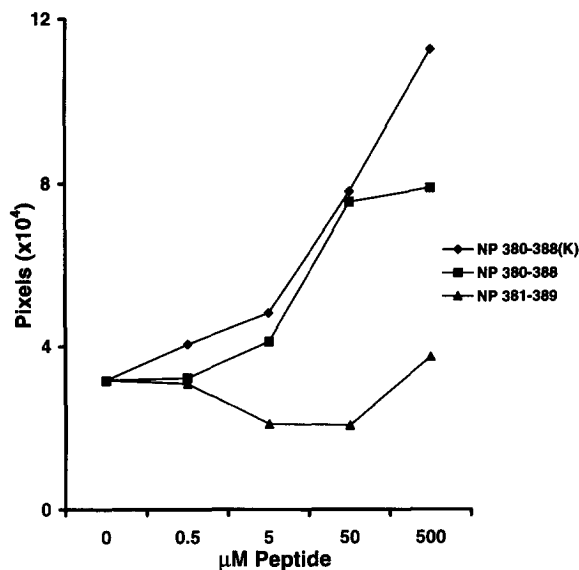


Figure 6. In Vitro Assembly of HLA-B8 with NP 380-388 Versus NP 380-388 (K)

Metabolically labeled HLA-B8 was assembled in the lysates of CHO cells using the various concentrations of peptide stated and then immunoprecipitated. Samples were analyzed on 12% NaDodSO₄-polyacrylamide gels and then quantitated using a phospho-imager. Pixels contained in the heavy chain band were plotted against peptide concentration. The NP 381-388 peptide binds B8 poorly because of its charged C terminus and is included as a negative control.

on the ability of B8 to bind the peptide (Figure 6). Note that inclusion of NP residue 389 completely abrogates binding of the peptide. This residue is charged and B8 prefers hydrophobic C termini (Sutton et al., 1993; DiBrino et al., 1994). In addition to these stabilization assays, we have also compared the titration curves for the 382R and 382K peptides in B8-restricted NP-specific CTL assays and find, using four different cell lines as targets, that the titration curves for the two peptides are virtually identical (data not shown). We therefore conclude that the lysine substitution alters the relative ability of the peptide to bind the two molecules by reducing its ability to bind B2702 and, as such, may be sufficient to restore presentation through B8 in NW cells.

A Lysine Substitution at Residue 382 Restores B8 Presentation in NW Cells

To test the hypothesis that B8 and B2702 compete for an NP peptide at the level of binding, recombinant vaccinia were generated that expressed either the wild-type NP sequence corresponding to residues 374-393 or this sequence with a lysine substitution at residue 382. Figures 7A-7D shows the data from four independent experiments using four different B8-restricted NP-specific CTL clones (established from two donors). Targets were NW and HW cells infected with either of these constructs. In all four experiments, peptide-pulsed HW and NW targets were killed comparably, and for clarity these data have been omitted from each graph. For lysis of the vaccinia targets,

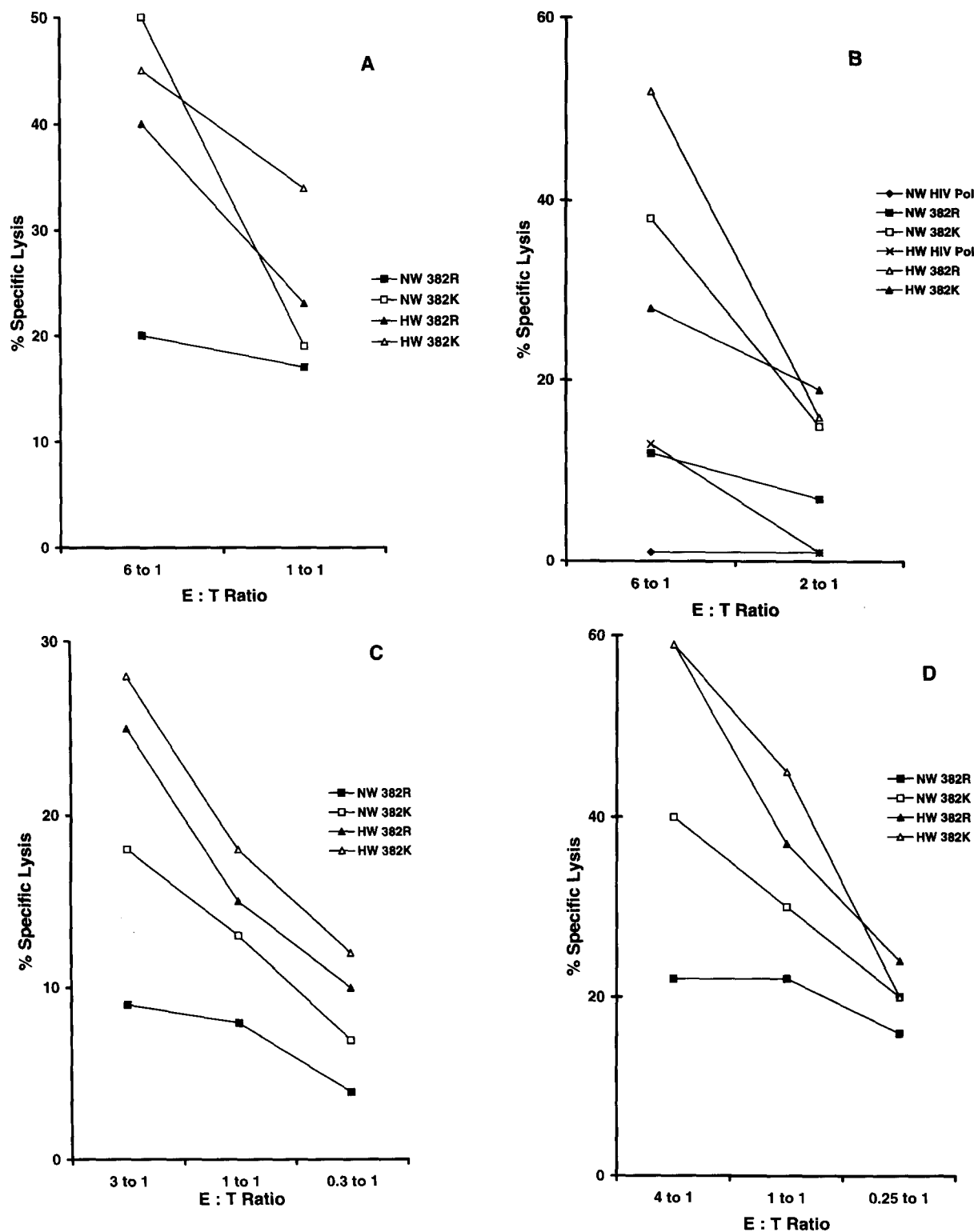


Figure 7. A Lysine Substitution at NP Residue 382 Restores Presentation of Viral-Derived NP 380–388 in NW Cells

(A–D) Targets were infected with recombinant vaccinias expressing either NP residues 374–391 without (382R) or with (382K) a lysine substitution at residue 382 or with HIV-Pol as an irrelevant control. The control B-LCL HW was established from the daughter of NW, who expresses B8 in combination with B44. Effectors are (A) CTL clone HW18 established from donor HW, (B) CTL clone HW6 established from donor HW, (C) CTL clone AR3 established from donor AR, and (D) CTL clone AR100 established from donor AR. Targets were lysed appropriately with added peptide, although for clarity the data has been omitted; lysis of targets in the absence of peptide was less than 5% for all ratios in each experiment. Lysis of targets infected with HIV-Pol was less than 3% for (A), (C), and (D) and was omitted for clarity.

however, we find that lysis of NW cells infected the 382K construct is substantially better than for those infected with the wild-type 382R construct, while lysis of virally infected HW cells is comparable using the two constructs. Since the recombinant vaccinias encode NP residues 374–393, it is formally possible that introduction of the lysine at position 382 alters processing in such a way as to now favor production of the B8 epitope. We feel that this is unlikely, since presentation through B8 is the same for both recombinant vaccinias in cells lacking B2702. We therefore interpret these results to mean that B8 and B2702 do in fact compete, at the level of binding, for a common fragment in the ER. This leads to suboptimal loading of B8 and the subsequent poor presentation of viral-derived antigen. A substitution that reduces the ability of B2702 to bind the peptide can give the competitive edge to B8, though, and restore presentation of viral-derived antigen.

Discussion

MHC class I molecules select peptides according to the specificity of their binding grooves, and this is believed to be the major determinant in the selection of antigenic peptides for presentation. Indeed, class I molecules are highly involved in determining which peptides occur intracellularly (Rotzschke and Falk, 1991). Sequencing of peptides eluted from class I molecules from virally infected cells has shown that epitopes can only be detected in cells that express their restricting molecules (Falk et al., 1990), and it is thought that protection of these epitopes by binding is the basis of such influence on the cellular peptide composition. Further, transfections of class I molecules from one species into the cells of another species allows presentation of both viral and alloptides restricted by that allele (Wallny et al., 1992; Rotzschke et al., 1991; Lobigs and Mullbacher, 1993), demonstrating that the presence of the restricting allele alone is sufficient for the appropriate processing and presentation of antigens, even across the species barrier. It is, therefore, unusual (provided that the processing and transport processes are intact) for a cell line that expresses the correct class I molecule to be unable to present an expected viral-derived epitope to CTL.

In previous studies, we described the donor NW whose cells differed from other B2702-expressing cell lines in that they failed to present certain peptide epitopes, whereas the other cell lines did (Pazmany et al., 1992). The failure to present through B2702 extended to several expected epitopes and occurred either following viral infection or with the addition of peptide. These earlier studies established that the B2702 molecule of NW was of normal sequence (Pazmany et al., 1992), and this along with data from other studies (Lopez et al., 1994; S. R.-J., unpublished data), suggested that compared with B2702 molecules in other cells, the B2702 molecules in the cells of NW may be assembling with a different subset of peptides. In subsequent studies of antigen presentation through HLA-B8, the NW cell line failed to present an expected epitope from the influenza A nucleoprotein, although

added peptide was presented normally (Rowland-Jones et al., 1993). The findings for antigen presentation through B8 differ from those for B2702 in certain respects, though. First, the antigen presentation polymorphism through B2702 extends to all family members carrying the HLA-A3 B2702 haplotype, while cells from the daughter of NW, who inherited the HLA-B8 haplotype, in the absence of the HLA-A3 B2702 haplotype present NP 380–388 following viral infection normally. Second, the failure to present following viral infection through B8 appears to be specific for the NP 380–388 peptide. And, finally, the NW cell line can present NP 380–388 when exogenously delivered. Thus, it appeared as though the NW cell line was specifically unable to generate the NP 380–388 peptide. If the same mechanism were responsible for the presentation polymorphisms through each of these alleles in NW and his family, then these data would suggest that a genetic factor, possibly linked to the HLA-A3 B2702 haplotype, was modifying antigen processing, selection, or both in their cells.

We now have investigated the molecular basis of the unusual properties associated with B8-restricted antigen presentation in the NW cell line and we find that, although we do not know the basis of the B2702 abnormality, different mechanisms are probably responsible for the properties of each allele. Mapping of a B2702-restricted influenza A epitope revealed a cluster of epitopes in the nucleoprotein, and within this cluster the B8 and B2702 epitopes overlap by 8 aa. Using a panel of B-LCL coexpressing B8 and B2702, we find that the NW phenotype (for B8 presentation) extends to other cell lines expressing the allelic combination of B8 and B2702. Further using transfectants of the class I-reduced cell line, C1R, we have demonstrated that this phenotype can be recreated by coexpressing B8 and B2702 in the same cell. Because the inability to present NP 380–388 is a function of the allelic combination of the cell, it is likely to be due to an event in the ER and the overlapping epitopes suggests competition between the alleles for a common peptide fragment. Deng et al. (1993) have described a similar mechanism for MHC class II alleles, although in this instance the competition appeared to be for a longer peptide fragment that spanned two disparate epitopes.

Several lines of evidence suggest that B8 has an apparent low affinity for the NP 380–388 peptide, even though it is an epitope that stimulates a vigorous CTL response in nearly all B8-positive individuals. In our lysate binding assays, levels of peptide binding were, overall, low. For other class I-peptide systems (i.e., HLA-A2 and Matrix 58–66 or HLA-A2 and HIV Pol), we can detect specific binding of peptide in the 1–10 nmol range using this assay (Tussey et al., 1994). With B8 and the NP peptide, however, we do not detect binding until the 100 nmol to 1 μ M range. Levels of peptide binding to B8 were also low in the transport assays and were approximately 30- to 40-fold lower than those seen with other class I-peptide systems. Two other lines of evidence support the idea that NP 380–388 has a relatively low affinity for B8. One is that the optimized B8 epitope does not titer past 10^{-9} M in CTL assays and is readily competed out by other B8 epitopes

(Sutton et al., 1993; S. R.-J. and L. T., unpublished data). The other derives from *in vitro* binding assays where class I complexes are reconstituted from free class I heavy chains, ^{125}I -labeled $\beta 2$, and peptide. The stability of these complexes can then be determined by monitoring $\beta 2$ dissociation on nondenaturing IEF gels. In such assays, the $t_{1/2}$ dissociation for most A2 antigenic peptides was 16–50 hr (A2 and Matrix 58–66 was 50 hr; Parker et al., 1992). The $t_{1/2}$ dissociation for B8 and NP 380–388, using this assay, however, is 2 hr (DiBrino et al., 1994). Our binding and transport studies further suggest that NP 380–388 binds B2702 almost as well as B8, but if we introduce a mutation in the epitope that alters the relative ability of the peptide to bind each allele, presentation through B8 is restored. Taken together, the data suggest that the consequence of the overlapping B8 and B2702 epitopes is that in a cell where both B8 and B2702 are assembling in the ER they compete for a common fragment, and this leads to suboptimal loading of B8. Thus, the failure of the NW cell line to present NP 380–388 is not due to any processing or selection polymorphism specific to the NW cell line, but rather to the fact that the cell coexpresses B2702 and B8. This, of course, fails to explain the unusual properties associated with B2702 presentation in these cells.

At first glance, it is difficult to imagine, given that sensitization for CTL recognition probably only requires 100–200 peptide–MHC complexes (Christinck et al., 1991), that competition for NP 380–388 by B2702 could actually be limiting for CTL recognition. Estimates of peptide copy numbers per cell, however, suggest that many peptides only occur at about 100–200 copies per cell (Rammensee et al., 1993). These estimates derive from analyses of class I-associated peptides and, as such, are a function of the ability of a given peptide not only to be processed but also on its ability to compete for translocation and then to compete for binding to its restricting allele. Thus, it is a series of processes and interactions that determines the number of complexes that are ultimately formed within the cell, and for many epitopes it appears that this number is just above the threshold required for CTL recognition. The NP 380–388 peptide is only a moderate competitor for translocation (Figure 5B), and this process could serve to limit the concentration of the peptide as it enters the ER. Peptides that are strong competitors for translocation characteristically have IC_{50} values in the range of 1 μM (Shepherd et al., 1993; Schumacher et al., 1994; Androlwicz and Cresswell, 1994). However, in NW cells NP 380–388 has an IC_{50} value of 30 μM , while the previously characterized B27 self-peptide (which is considered to be a strong competitor for translocation) has an IC_{50} of 0.2 μM . Certainly, other processes in the cytoplasm, such as processing of the epitope, or proteolysis of the peptide, could further serve to limit the concentration of this peptide. There is some evidence that limiting the availability of endogenously processed peptides can impair recognition by CTL (Milligan et al., 1990). We also find that NP 380–388 has an apparently low binding affinity for B8 (see above), and that this affinity is almost matched by that for B2702. Therefore, B2702 could compete almost as

effectively as B8 for these limiting concentrations of NP 380–388 in cells where they are simultaneously assembling, and this could lead to suboptimal loading of B8. Thus, we do not think that B2702 outcompetes B8 for the NP 380–388 peptide. But, rather, that there is a limiting amount of the NP peptide available for binding and that the simultaneous loading of this peptide onto B27 lowers the number of B8–NP complexes formed to below the threshold required for CTL sensitization. It is important to stress that B8 loading is suboptimal rather than nonexistent. We have observed, using certain CTL lines or with prolonged (>15 hr) CTL assays, that the competition for NP 380–388 by B2702 is not absolute. Therefore, we know that some B8–NP 380–388 complexes are being formed in NW cells and reaching the cell surface; apparently, though, under most circumstances the number of complexes reaching the cell surface is below the threshold required for CTL recognition. There are factors that could contribute to a situation where B2702 actually does outcompete B8 for the NP 380–388 peptide. For instance, B2702 could associate more efficiently with TAP than B8. Or for each allele NP 380–388 will be competing with a different pool of peptides for binding, and in this respect perhaps NP 380–388 competes relatively well for binding to B27 and less efficiently for binding to B8. At present, though, we favor the simpler model, whereby they both bind a peptide for which the concentration is limited.

For proteins in which large numbers of CTL epitopes have been mapped, it is clear that epitopes are not randomly distributed throughout the protein. Instead, they tend to cluster, and a fair number of overlapping epitopes have been reported (Johnson et al., 1992; Nixon et al., 1992; Elliott et al., 1993). This clustering may be due to the fact that most class I molecules bind peptides with hydrophobic anchors and often, because of structural and functional constraints, hydrophobic residues are clustered in proteins. Alternatively, it could reflect the proteolytic processes involved in the generation of antigenic peptides. It has been suggested that the secondary and tertiary structure of proteins may play a role in the generation of peptides, so that certain regions of the protein (depending on their orientation) are more effectively processed than others. The latter possibility would predict, though, that the position of the epitope in the protein would affect its ability to be generated. In at least one instance this seems to be the case (Del Val et al., 1991), although there are also instances where the position of the epitope has had no effect on the generation of the epitope (Chimini et al., 1989; Hahn et al., 1991).

The data presented here show that overlapping epitopes can influence epitope selection, but whether they will do so in each case is not clear. The effect of other overlapping epitopes, if any, on the processing, selection, or both, of epitopes really depends on how antigenic peptides are produced, about which little is known. One model put forth by Rotzchke and Falk (1991) incorporates the observation that most H-2 class I peptide motifs have a hydrophobic C terminus, and suggests that this may reflect the specificity of the proteolytic enzymes involved in generating antigenic peptides. With this model, the first step in antigen

processing is the generation of precursor fragments, varying in length, but with common C termini. Fragments fulfilling specific binding requirements bind the appropriate class I and trimming at the N terminus occurs. But, to date, there is little data as to the real nature of the fragments generated in the cytoplasm. Although TAP preferentially transports peptides of 8–11 aa, longer peptides are capable of being transported (Momburg et al., 1994a; Androlewicz and Cresswell, 1994); small populations of class I molecules associated with long peptides have been described (Joyce et al., 1994; Urban et al., 1994). These sorts of data, although indirect, do suggest that the proteolytic machinery indiscriminantly processes peptides to varying length in the cytoplasm, but those peptides of appropriate length for class I binding are preferentially transported. Given that shorter fragments will be preferentially represented in the ER, competition (at the level of binding) created by overlapping epitopes will probably be limited to epitopes of sufficient overlap as to be contained on a fragment of 8–11 aa.

Experimental Procedures

Subcloning of the HLA-B8 Gene

HLA-B8 cDNA cloned into the EcoRI (5')–BamHI (3') site of M13 was the gift of Dr. C. Quinn. The 5' EcoRI restriction enzyme site was changed to an XhoI site by first subcloning the cDNA into Bluescript (Stratagene). The gene was then subcloned into the XhoI–BamHI restriction sites of PREP-4 (Invitrogen).

Transfections and Maintenance of Cell Lines

HM2.C1R–B2702 (C1R–B2702) cells were the gift of Dr. J. Lopez de Castro (Madrid, Spain). The HLA-B8–PREP-4 construct was transfected into both the C1R and C1R–B2702 cell lines by electroporation. For this, 10^6 cells were mixed with 20 μ g of DNA in 0.5 ml of RPMI 1640 and electroporated using a gene (Bio-Rad Laboratories) electroporator set to 250 V and 500 μ F. Cells were resuspended in 50 ml of RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (R10) and plated in a 24-well plate. The next day, selective media containing 300 μ g/ml hygromycin B (CalBiochem) was added. Cell surface expression of the transfected B8 gene was determined by indirect immunofluorescence with Bw6 (Radka et al., 1982) and fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin G (IgG) followed by analytical flow cytometry. Positive transfectants were maintained in 300 μ g/ml hygromycin B. The HLA-B8–PREP-4 construct was introduced into CHO cells by electroporation with the electroporator set to 300 V and 960 μ F. Cells were plated as described above, except the selective and maintenance media contained 1 mg/ml hygromycin B. The human B lymphoblastoid cell lines, NW, HW, and HR were maintained in R10.

Generation of CTL Lines

Influenza-specific CTL lines were grown from peripheral blood mononuclear cells (PBMCs) as previously described (Gotch et al., 1987b; Nixon et al., 1988). They were maintained in culture by restimulation every 10–14 days with autologous irradiated (3000 rads) B-LCL, which had been pulsed for 1 hr with the appropriate peptide. The lines were cultured in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and 10 U/ml rat interleukin-2 (rIL-2) (Cetus Corporation, Emeryville, California).

Generation of CTL Clones

Peptide-specific CTL clones were generated from established CTL lines by seeding at 0.3 or 1 cell/well in flat-bottomed (HW clones) or (AR or HR clones) round-bottomed 96-well microtiter plates (Nunc, GIBCO) in 100 μ l of cloning mixture consisting of R10 containing mixed irradiated (3000 rads) PBMCs from three donors at a concentration of 10^5 per well, autologous irradiated (3000 rads) B-LCL (peptide

pulsed) at 10^4 per well, and phytohemagglutinin at a final concentration of 0.5 μ g/ml. On day 4, an additional 100 μ l of medium, supplemented with 20% lymphocult-T (Biotest) was added. Clones were expanded to 24-well plates after 11–14 days in a further 1 ml of cloning mixture and tested for specific activity. Positive clones were maintained in medium containing 10% lymphocult-T with weekly restimulations using autologous irradiated peptide-pulsed B-LCLs.

Generation of Recombinant Vaccinias

Purified oligonucleotides encoding NP residues 374–391 (MESSTLELRSSRYWAI RTR) or with the lysine substitution (MESSTLELKSRWAI RTR) were obtained from British Biotechnology (Oxford, England). These oligonucleotides included an NcoI site at the 5' end and a BglII site at the 3' end. Sequences of the oligonucleotides are the following: oligo A, 5'-CATGGAAAGTAGT ACTCTTAGCTCGTAGTCGTTATTGGG CTATTCGTA CTGTTAGCTGACTAGA-3' and its complementary strand, oligo B, 5'-GATCTCTAGTCAGCTAA CGAGTACGAATAGCCCAATAACG ACTACGAAGTTCAAGAGTACTACTT TC-3'; oligo C, 5'-CATGGAAAGTA GTACTCTGAACCTTAAGAGTCGTTATT GGGC-TATTCGTA CTGTTAGC TGACTAGA-3' and its complementary strand, oligo D, 5'-GATCTCTAGTCA GCTAACGAGTACGAATAGCCCAATAACGACTCTTAAGTTCAAGAG TA CTACTTTC-3'. For the annealing of the single-stranded oligonucleotides, 100 pmol each of oligo A and B or oligo C and D was heated to 95°C for 10 min and allowed to cool slowly to room temperature. The resulting double-stranded inserts were ligated into the NcoI and BglII sites of the transfer plasmid pSC11. The transfer vector was introduced into wild-type vaccinia virus infected TK⁻ 143 cells using lipofectin reagent (GIBCO BRL). Recombinant vaccinia were selected in the presence of BUdR and X-Gal. Recombinant plaques were taken through three rounds of purification. For overnight infections, 1×10^6 cells were infected with 1–2 pfu per cell for 1 hr at 37°C. Cells were washed and incubated overnight in 1 ml of culture medium. For daytime infections, 1×10^6 cells were infected with 3–5 pfu per cell for 1 hr at 37°C, washed, and incubated in 1 ml of culture medium for 3 hr.

Viruses and Peptides

Influenza A virus AX/31 containing the NP gene from the 1934 A/PR/8/34 strain of influenza was grown in the allantoic sacs of 11-day-old embryonated chicken eggs. Vac-imp encodes residues 1, 2, 327–498 of influenza A/NT/60/68 (Townsend et al., 1988). Peptides representing NP sequences were synthesized either using a Milligen/Bioscience 9500 automated peptide synthesizer and standard Merrifield tert-butoxycarbonyl chemistry (Dr. D. Klapper, University of North Carolina, Chapel Hill, North Carolina) or a Zinser analytic automated peptide synthesizer and standard 9-fluorenylmethoxycarbonyl chemistry. Peptides were high pressure liquid chromatography purified prior to use.

CTL Assays

Effector cells were tested for their ability to lyse ^{51}Cr -labeled target cells in a standard 4 hr ^{51}Cr release assay. Target cells, either B-LCLs or C1R transfectants, were labeled with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ for 1 hr at 37°C. Cells were washed twice in R10 and plated in wells of a round-bottomed 96-well plate at 5×10^5 cells/well in 150 μ l with or without CTL. For peptide assays, peptide was added to a final concentration of 10^{-7} M for each well. For assays of AX/31, antigen presentation targets were infected in serum-free medium with influenza virus 1–2 HAU per 100 cells as described (Gotch et al., 1987a). For assays with recombinant vaccinia viruses, 1×10^6 cells were either infected with 1–2 pfu/cell for 1 hr at 37°C, washed, and incubated overnight at 37°C in 1 ml of R10 or infected with 3–5 pfu/cell for 90 min at 37°C, washed, and incubated for 3 hr at 37°C. Chromium release was measured by harvesting 20 μ l of supernatant from each well onto a filtermat and counting in a β plate counter (LKB Instruments, Incorporated, Gaithersburg, Maryland). The results are expressed as per cent specific lysis, which is calculated as follows:

$$\text{Percent of specific lysis} = \left(\frac{\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spont}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{spont}}} \right) \times 100$$

Spontaneous release ($\text{cpm}_{\text{spont}}$) is determined from targets incubated

with medium alone and maximal release (cpm_{max}) is determined from targets incubated with 5% Triton X-100.

Iodinations

For the lysate binding assays, peptides were iodinated using a standard chloramine T assay. Peptide (10 µg) in 100 µl of phosphate-buffered saline was incubated for 60 s at 26°C with 1 mCi of ¹²⁵I (Amersham) and 10 µl of chloramine T (5 mg/ml). The reaction was stopped by adding 30 µl of sodium metabisulfite (5 mg/ml) and 200 µl of 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% NP-40, 0.1% bovine serum albumin, and 0.02% sodium azide. Unbound iodine was removed by passage over a Sephadex G-10 spin column. For the intracellular transport assays, peptides were iodinated as previously described (Androlewicz et al., 1993).

Peptide Binding Assays

Peptide binding assays were performed essentially as previously described (Tussey et al., 1994). In brief, 10⁷ cells were incubated for 45 min at 37°C in methionine-free medium. [³⁵S]Trans-label (120 µCi) (Amersham) was added and the cells were incubated for 1 hr at 37°C. Cells were washed two times in phosphate-buffered saline then lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% NP40. After 30 min on ice, lysates were cleared by centrifugation for 10 min in a refrigerated microfuge. ¹²⁵I-labeled peptide was added, usually over the range of 2 × 10⁶–8 × 10⁷ cpm, along with 90 µl of 10% Staphylococcus aureus immunoadsorbent. Based on the specific activity of the peptide, we estimate that with this range of cpm, labeled peptide was added to a final concentration of 50 nM to 2 µM in these assays. Samples were immunoprecipitated with 10 µg of the human class I-specific antibody W6/32 (Barnstable et al., 1978) and an excess of immunoadsorbent. Immunoprecipitates were washed three times in lysis buffer and analyzed on a 15% NaDodSO₄-polyacrylamide gel under reducing conditions. Gels were quantitated using a phosphorimager and the results reported as the ratio of pixels contained in the peptide band to those contained in the heavy chain band.

Assembly of HLA-B8 in CHO Cells

Cells (8 × 10⁷) were starved for 1 hr in 1 ml of methionine-free media. ³⁵S-Trans label (1.2 mCi) was added and cells were incubated at 37°C for 1–2 hr. Cells were washed and lysed as above, except that the lysis buffer also contained 5 µg/ml human β₂-microglobulin (Sigma). After 30 min on ice, lysates were cleared by centrifugation for 10 min in a refrigerated microfuge. The cleared lysate was aliquoted among tubes containing varying concentrations of peptide. We added 90 µl of 10% Staphylococcus aureus immunoadsorbent and the samples were rocked overnight at 4°C. Samples were immunoprecipitated, analyzed, and quantitated as above. The results are reported as the number of pixels contained within the heavy chain band.

Intracellular Loading and Transport Assays

Permeabilization of cells with streptolysin O and the intracellular loading assays were carried out as previously described (Androlewicz et al., 1993). In brief, 2 × 10⁷ cells were permeabilized and incubated with 2 × 10⁷ cpm of ¹²⁵I-labeled peptide. Based on the specific activity of the labeled peptide, the final concentration of peptide used in these assays is between 200 nM to 1 µM and is comparable to the concentration of peptide used in the lysate binding assays. Peptide translocation was allowed to proceed for 10 min, cells were then washed and solubilized in 1 ml of buffer containing 1% Triton X-100, 0.15 M NaCl, 0.01 M Tris-HCl (pH 7.4), 5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µM of the appropriate unlabeled peptide to prevent postsolubilization binding of the labeled peptide. Lysates were clarified by centrifugation at 10,000 × g for 5 min, immunoprecipitated with 10 µg of W6/32 (Barnstable et al., 1978), and the level of bound peptide determined by counting in a γ counter (LKB Compugamma CS). The peptide competition assays were essentially as previously described (Androlewicz and Cresswell, 1994). Basically, 10⁷ SLO-treated NW cells were incubated for 5 min at 37°C with various concentrations of unlabeled competitor peptides and ¹²⁵I-B27#3* (50 nM). B27#3* (RRYQNSTEL) is a variant of a B27 self-peptide, which has an N-linked glycosylation acceptor site and has been previously characterized in

these assays (Androlewicz and Cresswell, 1994). Cells were washed and solubilized as with the intracellular loading assays. The glycosylated ¹²⁵I-B27#3* peptide was isolated from the extracts using concanavalin A-Sepharose beads (25 µl, 50% suspension, Sigma). Pellets were washed and counted in a γ counter.

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